

is made nearly neutral. When so constructed, the presence or absence of a single phosphate group can mean the difference between a net negative or a net positive charge. This observation has particular utility when one objective is to discriminate between enzymatically generated fragments of DNA, which lack a 3' phosphate, and the products of thermal degradation, which retain a 3' phosphate (and thus two additional negative charges).

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**a) Characterization Of The Products Of Thermal Breakage Of DNA Oligonucleotides**

Thermal degradation of DNA probes results in high background which can obscure signals generated by specific enzymatic cleavage, decreasing the signal-to-noise ratio. To better understand the nature of DNA thermal degradation products, we incubated the 5' tetrachloro-fluorescein (TET)-labeled oligonucleotides 78 (SEQ ID NO:59) and 79 (SEQ ID NO:60) (100 pmole each) in 50 µl 10 mM NaCO<sub>3</sub> (pH 10.6), 50 mM NaCl at 90°C for 4 hours. To prevent evaporation of the samples, the reaction mixture was overlaid with 50 µl of ChillOut® 14 liquid wax (MJ Research). The reactions were then divided in two equal aliquots (A and B). Aliquot A was mixed with 25 µl of methyl violet loading buffer and Aliquot B was dephosphorylated by addition of 2.5 µl of 100 mM MgCl<sub>2</sub> and 1 µl of 1 unit/µl Calf Intestinal Alkaline Phosphatase (CIAP) (Promega), with incubation at 37°C for 30 min. after which 25 µl of methyl violet loading buffer was added. One microliter of each sample was resolved by electrophoresis through a 12% polyacrylamide denaturing gel and imaged as described in Example 21; a 585 nm filter was used with the FMBIO Image Analyzer. The resulting imager scan is shown in Figure 55. In Figure 55, lanes 1-3 contain the TET-labeled oligonucleotide 78 and lanes 4-6 contain the TET-labeled oligonucleotides 79. Lanes 1 and 4 contain products of reactions which were not heat treated. Lanes 2 and 5 contain products from reactions which were heat treated and lanes 3 and 6 contain products from reactions which were heat treated and subjected to phosphatase treatment.

As shown in Figure 55, heat treatment causes significant breakdown of the 5'-TET-labeled DNA, generating a ladder of degradation products (Figure 55, lanes 2, 3, 5 and 6). Band intensities correlate with purine and pyrimidine base positioning in the oligonucleotide sequences, indicating that backbone hydrolysis may occur through formation of abasic intermediate products that have faster rates for purines than for pyrimidines [Lindahl and Karlström (1973) Biochem. 12:5151].

Dephosphorylation decreases the mobility of all products generated by the thermal degradation process, with the most pronounced effect observed for the shorter products (Figure 55, lanes 3 and 6). This demonstrates that thermally degraded products possess a 3' end terminal phosphoryl group which can be removed by dephosphorylation with CIAP. Removal of the phosphoryl group decreases the overall negative charge by 2. Therefore, shorter products which have a small number of negative charges are influenced to a greater degree upon the removal of two charges. This leads to a larger mobility shift in the shorter products than that observed for the larger species.

The fact that the majority of thermally degraded DNA products contain 3' end phosphate groups and Cleavase® enzyme-generated products do not allow the development of simple isolation methods for products generated in the invader-directed cleavage assay. The extra two charges found in thermal breakdown products do not exist in the specific cleavage products. Therefore, if one designs assays that produce specific products which contain a net positive charge of one or two, then similar thermal breakdown products will either be negative or neutral. The difference can be used to isolate specific products by reverse charge methods as shown below.

**b) Dephosphorylation Of Short Amino-Modified Oligonucleotides Can Reverse The Net Charge Of The Labeled Product**

To demonstrate how oligonucleotides can be transformed from net negative to net positively charged compounds, the four short amino-modified oligonucleotides labeled 70, 74, 75 and 76 and shown in Figures 56-58 were synthesized (Figure 56

shows both oligonucleotides 70 and 74). All four modified oligonucleotides possess Cy-3 dyes positioned at the 5'-end which individually are positively charged under reaction and isolation conditions described in this example. Compounds 70 and 74 contain two amino modified thymidines that, under reaction conditions, display 5 positively charged R-NH<sub>3</sub><sup>+</sup> groups attached at the C5 position through a C<sub>10</sub> or C<sub>6</sub> linker, respectively. Because compounds 70 and 74 are 3'-end phosphorylated, they consist of four negative charges and three positive charges. Compound 75 differs from 10 74 in that the internal C<sub>6</sub> amino modified thymidine phosphate in 74 is replaced by a thymidine methyl phosphonate. The phosphonate backbone is uncharged and so there are a total of three negative charges on compound 75. This gives compound 75 a net negative one charge. Compound 76 differs from 70 in that the internal amino 15 modified thymidine is replaced by an internal cytosine phosphonate. The pK<sub>a</sub> of the N3 nitrogen of cytosine can be from 4 to 7. Thus, the net charges of this compound, can be from -1 to 0 depending on the pH of the solution. For the simplicity of analysis, each group is assigned a whole number of charges, although it is realized that, depending on the pK<sub>a</sub> of each chemical group and ambient pH, a real charge may differ from the whole number assigned. It is assumed that this difference is not significant over the range of pHs used in the enzymatic reactions studied here.

Dephosphorylation of these compounds, or the removal of the 3' end terminal 20 phosphoryl group, results in elimination of two negative charges and generates products that have a net positive charge of one. In this experiment, the method of isoelectric focusing (IEF) was used to demonstrate a change from one negative to one positive net charge for the described substrates during dephosphorylation.

Substrates 70, 74, 75 and 76 were synthesized by standard phosphoramidite 25 chemistries and deprotected for 24 hours at 22°C in 14 M aqueous ammonium hydroxide solution, after which the solvent was removed in vacuo. The dried powders were resuspended in 200 µl of H<sub>2</sub>O and filtered through 0.2 µm filters. The concentration of the stock solutions was estimated by UV-absorbance at 261 nm of samples diluted 200-fold in H<sub>2</sub>O using a spectrophotometer (Spectronic Genesys 2, 30 Milton Roy, Rochester, NY).